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OBSERVATIONS ON MOLECULAR WEIGHT ESTIMATIONS IN POLYACRYLAMIDE GELS

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SUMMARY

A study was made of the relative retardation of low-molecular-weight proteins in polyacrylamide gel electrophoresis at pH 4.5. It was found that low-molecular-weight proteins need not be separated from each other and may migrate with the Pyronin Y dye front in different lower percentage acrylamide gels. This behavior of low-molecular-weight proteins is examined with regard to ambiguities in standard plots which are currently used to determine the molecular weight of unknown proteins.

INTRODUCTION

A technique in protein biochemistry which is growing in use is the Hedrick and Smith[1] method of estimating the molecular weight of proteins by polyacrylamide gel electrophoresis which was based on the observation that, following electrophoresis in gels of differing acrylamide concentration, there was a linear relationship between retardation coefficient (K_R) and protein molecular weight (MW) between 45,000 and 500,000. The linear relationships of such plots (K_R *vs.* MW) have since been modified and changed to include linear plots of the square root of retardation coefficient *versus* molecular radius ($\sqrt{K_R}$ *vs.* $\bar{R}$) (Refs. 2–4). The simplicity of the Hedrick and Smith[1] method and the fact that it is accomplished without denaturing agents such as sodium dodecyl sulphate has resulted in it being widely used to estimate the molecular weight of mixtures of proteins[5–10] and multiple forms of enzymes[11,12]. Among other linear relationships developed to estimate molecular weights of proteins by gel electrophoresis has been a ratio of relative mobilities to log molecular weight where linearity was found from 60,000 to 160,000 molecular weight[13]. In some reports the standard curves of Hedrick and Smith (K_R *vs.* MW) have been extrapolated linearly to a retardation axis[7–10] suggesting that even the size of small proteins can be estimated directly by this method and implying that as the size of a molecule approaches zero that it still possesses a retardation coefficient. Rodbard and Chrambach[2,3] provided a mathematical theory to show that low-molecular-weight material would be retarded in all gels.

In this report the standard curve relating retardation coefficient to molecular weight has been investigated for low-molecular-weight proteins to see whether or not low-molecular-weight proteins possess a retardation coefficient. The molecular weight estimation method reported here is at acidic conditions and is applicable to any

protein or enzyme for which there is a specific stain available and which migrates on polyacrylamide gels under the acidic conditions employed by Reisfield *et al.*[14]. Included in this report is an account of some technical difficulties encountered in determining molecular weights of proteins by polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Proteins

The following proteins were used in these studies: Ovalbumin (45,000) and ribonuclease A (13,700) (L.K.B., Stockholm, Sweden) and human γ-globulin (160,000), albumin (65,000), α-chymotrypsinogen (25,000), myoglobin (17,800) and cytochrome C (12,500) (Schwarz-Mann, Orangeburg, N.Y., U.S.A.) were obtained from the kits of marker proteins supplied to calibrate gel filtration columns. The same proteins and lysozyme (14,500) and hemoglobin (64,000) were obtained separately from Sigma (St. Louis, Mo., U.S.A.) or Calbiochem (Los Angeles, Calif., U.S.A.).

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed essentially as described by Reisfield *et al.*[14], except that the β-alanine–acetic acid tray buffer at pH 4.5 was diluted to 1/3 strength for use in the electrode reservoirs. Gels of different acrylamide concentrations were used, as described by Hedrick and Smith[1], for the electrophoresis of acidic proteins. The running gel was 0.5×10 cm, and was polymerized for 90 min with 0.14% (w/v) ammonium persulphate. The stacking gel was omitted and the different percent (w/v) acrylamide gels were prepared from a stock solution containing 0.735 g N,N'-methylenebisacrylamide and 40 g acrylamide in 100 ml water. A Bio-Rad Model 150 electrophoresis cell (Bio-Rad Lab., Richmond, Calif., U.S.A.) was used in conjunction with a Büchler (Fort Lee, N.J., U.S.A.) power supply and electrophoresis was conducted at 0 to 4°. Generally for each electrophoretic run, duplicate samples were run on either 5–10% or 10–17% (w/v) acrylamide gels. Samples diluted in 10% (w/v) sucrose were applied to the top of the running gel and 10 μl of 0.1% (w/v) Pyronin Y (Eastman-Kodak, Rochester, N.Y., U.S.A.) was used as a tracking dye. Electrophoresis was conducted at 4 to 5 mA per tube until the Pyronin Y marker had migrated to within 1 cm of the end of the lowest percent acrylamide gel. The gels were fixed and stained with Coomassie Blue as previously described[11].

Treatment of data

Relative mobility, defined as the ratio of the distance moved by each protein band to the distance moved by Pyronin Y included with the sample when applied to the gel, was measured as previously described[11] and the data treated as in Hedrick and Smith[1] or in Rodbard and Chrambach[2,3]. Each protein was identified by subjecting it independently to electrophoresis in different percentage acrylamide gels. Proteins were distinguished from contaminants by comparing samples from different commercial sources. For complex mixtures of proteins the identity of a particular band from one gel to another was followed both by the intensity of staining in the gels and by the observation that, in general, plots of log relative mobility *versus* gel concentration are linear[3].

RESULTS

Fig. 1 shows the effect of gel concentration on the electrophoretic mobilities of ovalbumin, α-chymotrypsin, myoglobin and cytochrome C. The gels have been loaded so as to emphasize contaminating or artifactual bands which appear in the gels. Cytochrome C in these gels remains at the dye front and is not unstacked from the dye front unless gels above 14% (w/v) acrylamide are employed.

In obtaining the standard curve to determine molecular weights by polyacrylamide gel electrophoresis, purer protein samples are needed than those used to calibrate gel filtration columns, since minor contaminants which remain unnoticed following gel filtration upset identification following electrophoresis. Ovalbumin (L.K.B.) and γ-globulin (Schwarz-Mann) in particular gave multiple protein bands following electrophoresis although these samples are supplied in protein marker kits for calibrating gel filtration columns. Two components have been previously noted on the electrophoresis of samples of ovalbumin[4]. Again the true protein band for the

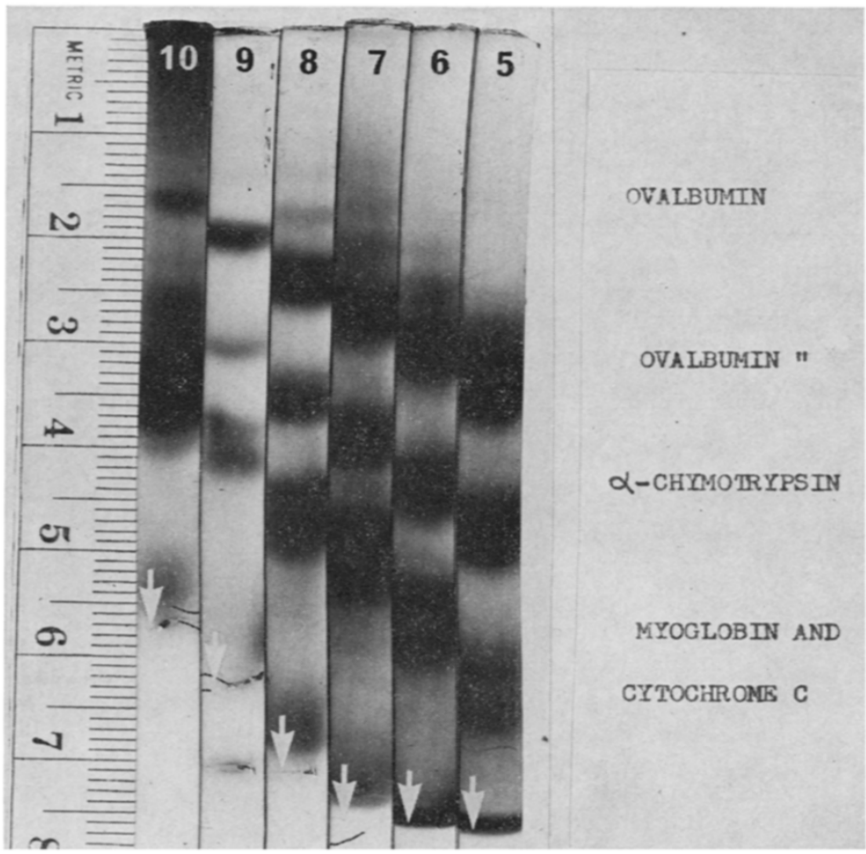

Fig. 1. The effect of 10–5% (w/v) acrylamide gel concentrations, from left to right, on the mobility of ovalbumin, an extra component seen on the electrophoresis of ovalbumin (ovalbumin") together with α-chymotrypsin, myoglobin and cytochrome C. Electrophoresis at pH 4.5 was toward the cathode at the bottom of the picture: The final position of the dye marker, Pyronin Y, is indicated by an arrow in each gel. Experimental details are described in the text.

protein under investigation was identified by comparison of samples from different commercial sources.

To obtain Ferguson plots of relative mobility *versus* gel concentration, as obtained by Hedrick and Smith[1], it was critical that (i) electrophoresis be carried out in gels above 5% (w/v) acrylamide concentration, (ii) the relative mobility lay between 0.1 and 0.9, and (iii) the polymerization time and temperature be kept constant[3,4]. Polymerization times beyond 2 h led to changes in the gel pores since the marker dye no longer migrated as a discrete band and this was also observed if the discontinuity of the system was disturbed by performing pre-electrophoresis.

Fig. 2 shows the negative slopes obtained from log plots of relative mobility

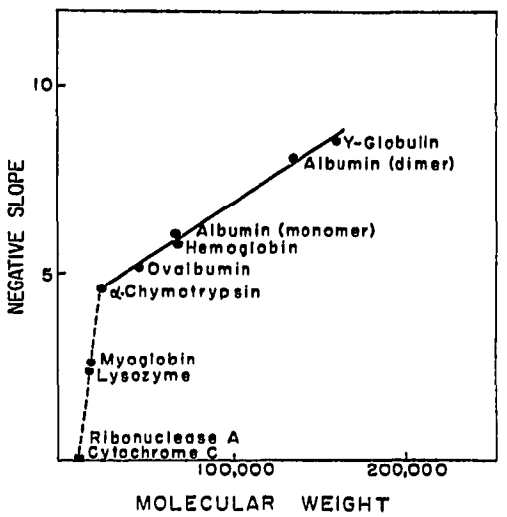

Fig. 2. The relationship of the negative slope (K_R) to the molecular weight of standard proteins subjected to polyacrylamide gel electrophoresis in 5–10% (w/v) acrylamide gels, at pH 4.5 using Pyronin Y as a tracking dye.

versus gel concentration for some standard proteins following electrophoresis at pH 4.5 in 5–10% (w/v) acrylamide gels. Between 25,000 and 160,000 molecular weight a linear relationship was obtained with respect to slope or retardation coefficient. Below 25,000 molecular weight a non-linear relationship was obtained so that for small slope values the standard plot was best represented by a curve.

The line relating retardation coefficient to molecular weight intercepted on the molecular weight axis when calibration was performed on 5–10% (w/v) acrylamide gels. This is illustrated in Fig. 2 by the zero slopes shown for ribonuclease A and cytochrome C, both of which migrated with the dye front under the conditions used here and which were indistinguishable from each other, except for the color given by cytochrome C, if both were run together on the one 5–10% (w/v) acrylamide gel.

This migration of small proteins with the dye marker is shown in 5 and 6% gels in Fig. 1, where myoglobin and cytochrome C are indistinguishable from each other on these gels. The accumulation of low-molecular-weight proteins at the dye front becomes a problem in establishing slope in fairly large-pore gels. The small slope for

TABLE I

RETARDATION COEFFICIENTS OF STANDARD PROTEINS

No.	*Protein*	*Hedrick–Smith method*[1]			*Rodbard–Chrambach method*[3]	
		Molecular weight	*Negative slope or retardation coefficient*		*Molecular radius*	*Square root of retardation coefficient* ($\sqrt{K_R}$) *with 10–17% gels*
			5–10% gels	*10–17% gels*		
1	Cytochrome C	12,500	0.0	2.9	1.537	0.1702
2	Ribonuclease A	13,700	0.0	2.9	1.550	0.1702
3	Lysozyme	14,500	2.4	3.8	1.620	0.1949
4	Myoglobin	17,800	2.6	3.9	1.734	0.1974
5	α-Chymotrypsin	25,000	4.6	4.4	1.945	0.2097
6	Ovalbumin	45,000	5.4	5.2	2.336	0.2280
7	Hemoglobin	64,000	5.8	5.7	2.664	0.2387
	Albumin					
8	Monomer	67,000	6.0	5.9	2.698	0.2428
9	Dimer	134,000	7.9	7.4	3.400	0.2720
10	γ-Globulin	160,000	8.5	7.9	3.607	0.2810

myoglobin shown in Fig. 2 was obtained from the plot of log mobility *versus* gel concentration in 8–10% (w/v) acrylamide gels since this protein ran with the dye front in lower percent gels. A similar situation held for lysozyme. These proteins were distinguishable from each other by using gels of higher percent acrylamide concentration but cytochrome C and ribonuclease A could not be distinguished in any 5–10% (w/v) gel under our conditions of electrophoresis.

Gels above 14% (w/v) acrylamide were needed to unstack ribonuclease A and cytochrome C from each other and these proteins then possessed retardation coefficients following electrophoresis in these smaller-pore gels (Table I). The retardation coefficients obtained for the low-molecular-weight proteins following electrophoresis in 10–17% (w/v) acrylamide gels have the effect of raising and shifting slightly to the ordinate, the non-linear portion of the calibration curve shown in Fig. 2 but this curve inflects toward the molecular weight axis.

A similar inflection of the standard curve was obtained when the data in Table I were plotted as $\sqrt{K_R}$ *vs.* $\bar{R}^3$, where $\bar{R}$ is the molecular radius (Fig. 3). Note that for this plot, the retardation coefficient is taken as one hundredth of the K_R values of Hedrick and Smith. The plot of $\sqrt{K_R}$ *vs.* $\bar{R}$ gave a better fit than K_R *vs.* MW for the relationship between protein size and retardation coefficient over the linear range, suggesting that the molecular radius, rather than molecular weight, may be a more appropriate basis to estimate protein molecular size by polyacrylamide gel electrophoresis[2–4]. The linear relationship obtained for $\sqrt{K_R}$ *vs.* $\bar{R}$ suggests that in this work it was possible to neglect the small amount of monomer used, since another difference between the Hedrick–Smith[1] and Rodbard–Chrambach[3] methods is that the latter calculates acrylamide percentage from the total acrylamide concentration rather than the amount of monomer. However, it was noted that a plot of $\sqrt{K_R}$ *vs.* $\bar{R}$, for the data obtained with 5–10% (w/v) gels, gave a curve intercepting on the molecular radius

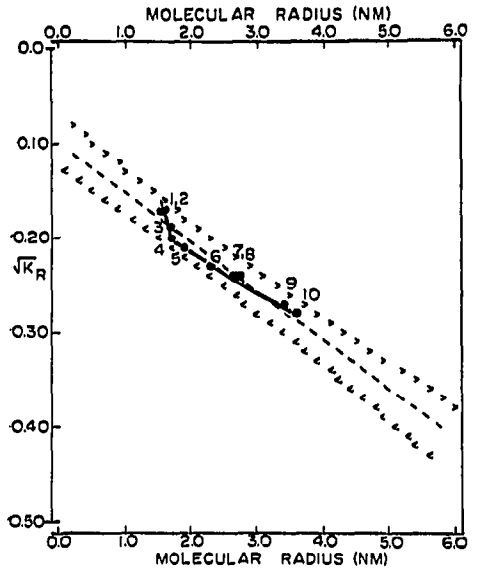

Fig. 3. The relationship of $\sqrt{K_R}$ to the molecular radii of the standard proteins listed in Table I when subjected to polyacrylamide gel electrophoresis in 10–17% (w/v) acrylamide gels, at pH 4.5 using Pyronin Y as a tracking dye (——). The dashed line represents a computer-fitted linear regression analysis through all points with the 95% confidence limits indicated by brackets: The validity of drawing this line is discussed in the text.

axis while the inflection noted in the plot of $\sqrt{K_R}$ *vs.* $\bar{R}$ for the data obtained in the 10–17% gels (Fig. 3) did not permit a linear regression extrapolation of all points to the retardation axis as done by Rodbard and Chrambach[2,3]. In Fig. 3 such a computer-estimated extrapolation is shown by the broken lines, with the 95% confidence limits indicated by the brackets, performed in the manner of Rodbard and Chrambach[3]. However, no justification for this linear extrapolation was forthcoming to suggest that as the molecular radius decreased to zero a molecule would still possess a retardation constant.

DISCUSSION

The main conclusion from this work is to emphasize that small-molecular-weight proteins need not be separated from each other by polyacrylamide gel electrophoresis and may migrate with the dye or migrating ion front in different percentage acrylamide gels. In this work, when comparatively large-pore 5–10% (w/v) acrylamide gels were used for electrophoresis of proteins below 25,000 molecular weight, there was a non-linear relationship in both the Hedrick–Smith[1] and Rodbard–Chrambach[3] plots of retardation coefficient *versus* molecular size. It is suggested from this work that serious discrepancies can occur in estimating molecular weights by these methods if it is not realized that the line relating retardation coefficient (K_R or $\sqrt{K_R}$) to molecular size intercepts or inflects toward the molecular weight/radius axis; all sizes below the point of interception will not be separated from each other and so will be indistinguishable in such a calibrated system. A consequence of this

observation is that low molecular-weight proteins, whose homogeneity has been determined by polyacrylamide gel electrophoresis under conventional conditions, may have to be re-examined for purity using either higher percent acrylamide gels or gels with lower bisacrylamide concentrations.

Under the conditions described here for 5–10% acrylamide gels the system has certain advantages, for it would appear to be particularly easy to distinguish between proteins of molecular weight below 10,000, assuming that they migrate, and proteins above 25,000 molecular weight, since the former would migrate with the Pyronin Y dye marker while those of higher molecular weight would be retained in the gels.

At least one report on the estimation of the size of low-molecular-weight proteins by polyacrylamide gel electrophoresis has suggested that the Hedrick–Smith plot can be drawn as an exponential curve for proteins of size 10,000 to 50,000 molecular weight[15]. Under the conditions of electrophoresis used by Felgenhauer[16], proteins having Stokes radii smaller than 30 Å were found not to be retarded below a certain acrylamide concentration, a result that was attributed to the molecules being below the "retardation limit" of the gels.

For the standard proteins which fell on the linear calibrated curve in this work, the variation in value for molecular weight from one run to another was in the order of ±20%. There appears to be little validity in extrapolating this linear curve to the retardation axis, as erroneously done by one of us in estimating the size of an "anti-band"[11], and considerably higher errors (±100%) can occur in estimating molecular weights outside the range of calibration, as has also been done in other reports[7–10,17]. The analogy between gel filtration and gel electrophoresis has been made and examined[3,4]. From this report it is suggested that, as in gel filtration, it is unwise to deduce molecular weights by extrapolation of the calibrated standard curve. Some reports have investigated the upper exclusion limits of the gels[4,18]. The results of this work on the lower limits of polyacrylamide gels employed for electrophoresis suggest that the analogy between gel electrophoresis and gel filtration is also similar in that there will be small-molecular-weight materials which, because they are too small to be fractionated by the gel, will not be separated from each other.

ACKNOWLEDGEMENT

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